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13. ABSTRACT (Maximum 200 words)

Sepsis from bacterial contamination of blood is an infrequent event in transfusion medicine; however, such an event may lead to a fatal outcome or to other serious consequences. Currently, a rapid, non-invasive, low technology method for the detection of bacterial contamination in banked blood is not available for routine use. The purpose of the work undertaken during Phase I of this project was to examine the feasibility of applying a remote sensor to the detection of a common bacterial metabolite as a marker for microbial contamination of banked blood. Upon spiking whole blood with a series of 10 bacterial species each able to generate the marker at 4^{0} C, a simple remote sensor device was found to change color. The results obtained from this Phase I effort provide scientific proof in support of the concept that a remote colorimetric sensitive sensor can be applied to the detection of microbial contaminants in banked blood.

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David Feldenstein August 25
PI - Signature Date
1994

(4) Table of Contents

Phase I Final Report

Systems to Detect Bacterial Contamination of banked Blood in a Rapid, Non Invasive, Low Technology Manner

FINAL REPORT

(5) Introduction.

a. *Nature of Problem:* Sepsis from bacterial contamination of blood is an infrequent event in transfusion medicine; however, such an event may lead to a fatal outcome or to other serious consequences (1). Currently, a rapid, non-invasive, low technology method for the detection of bacterial contamination in banked blood is not available for routine use.

A major technical problem of detecting bacterial contamination in whole blood is avoiding inadvertent (additional) contamination through the analytical process or measurement--*invasiveness of integrity*; finding a marker or series of markers universally present in numerous potentially contaminating bacteria which is absent in blood and blood components--*exclusivity*; performing a candidate indicator test system rapidly or continuously with accumulating signaling--*a tell-tale sensor*; plus performing the detection at low cost considering the large number of units collected--*economical*. Also, due to the large numbers of collected blood processed into components, inventoried and storage maintained, it is advantageous to consider a signaling system which is capable of automated detection through unskilled operation--*unattended operation*.

b. Previous Work: Methods for the detection of microorganisms in liquid media described in the literature involve the employment of costly equipment, inoculation of samples into a growth medium (invasiveness), significant time periods for incubation and occasionally, the utilization of radioactive reagents. As one example, U.S. Patent No. 5,232,839 (2), describes a method and apparatus that monitors the rate of change over time in the pressure within the headspace above a sample in a closed vessel to provide an indication of microbial growth in the sample. Other methods include:

❒ *Microscopy.* The microscopic evaluation of Gram-or acridine orange-stained blood smears has been considered a bacterial screening test to be performed just before transfusion. Because of the high limits of reproducibly detecting bacteria and the labor intensity of the method, these have been considered poor screening techniques (8).

❒ *Culture methods.* Instrumented blood culturing methods resulting in rapid detection of bacteria by measuring increasing levels of carbon dioxide released by proliferating bacteria have been tested. These methods are time consuming and may not be effective in detecting bacteria in freshly drawn units when contamination is low(9).

❒ *Nucleic acid hybridization.* Has fast results using a "universally conserved" bacterial rRNA and detection limits of 10^4 CFU/ml. This test could potentially be performed just before transfusion(10).

❒ *PCR.* PCR-based methods have been developed but current problems include arduous nucleic acid preparation, extraneous contamination, and non-universal bacterial signal detection(11).

c. *Purpose of Present Work:* The purpose of the work undertaken during Phase I of this project was to examine the feasibility of applying a remote sensor to the detection of a common bacterial metabolite as a marker for microbial contamination of banked blood.

d. *Methods of Approach:* The remote sensor, ammonia-developing diazo paper or film, was affixed to the inner surfaces of the screw caps of sterile plastic vials. These materials are sensitive to low levels of ammonia, a common microbial metabolite, and visibly change color from light yellow to black when exposed to low levels of ammonia. Banked blood alone (controls) or blood spiked with ammonium carbonate; cell-free ammonia generating enzymes from bacteria and plant sources; or one of ten selected bacterial species were incubated at 4^{O} C for varying time periods. Sensor disks were then examined visually for a color change to black (yes/no) and then by Laser Scanning Densitometry for quantitation of the color changes.

(6) Narrative:

a. Experimental Methods**:**

1. Bacteria: Ten bacterial species, representing a diversity of metabolic pathways for ammonia production, were obtained from the American Type Culture Collection (Appendix pA1). Among these species, some produce ammonia by action of the enzyme urease on urea (3, 4) , others evolve ammonia by cleavage of arginine by the enzyme arginine dehydrolase, and the remainder by catabolic pathways involved in protein and peptide degradation (5, 6). Lyophilized ATCC culture preparations were reconstituted in Tryptone broth, streaked to agar media to check for purity, tested with API Diagnostic kits to validate species identity and maintained as stocks on Tryptone agar slants stored at 4^{O}C. Working cultures for the experiments described below were initiated from inocula from stocks to fresh Tryptone broth incubated overnight (18 to 20 hours) at 37^{O} C.

2. Remote Sensor System: A variety of ammonia sensitive diazoprint papers and films were obtained commercially (Azon Corporation, formerly Post & Company). Disks, approximately 1/4" in diameter, were cut from these materials and affixed to the inner surfaces of the screw caps to sterile plastic cryovials or cut into rectangular sheets for placement over microtiter plates which had been charged with small volumes of bacterial and substrate mixtures.

3. General Experimental Procedures:

a. Test Systems Employed:

1. Preliminary Screening Experiments: Microtiter plates containing 96 x 300 µl wells were utilized as a screening device in preliminary experiments designed to detect ammonia evolution from bacterial-aqueous substrate mixtures. In these screening experiments, 10 µl of the bacterial suspensions were added with mixing to 50 µl of aqueous substrate. The microtiter plates were then overlaid with rectangular sheets of ammonia-sensitive blueprint paper or film leaving a head space of approximately 10 mm between the paper or film and the liquid sample surfaces in the charged wells. The plates were then incubated for periods of up to 7 days at ambient room temperature or at 4^{o} C. Following incubation the ammonia-sensitive papers and films were removed and examined for the development of black spots above the sample wells.

2. Blood-Microbial Interactions: Sterile NUNC 5 ml screw-capped plastic cryovials were used to evaluate the remote sensor system in experiments designed to study ammonia evolution generated from bacterial contamination of sterile fetal calf serum or banked blood. In these experiments 0.2 ml of bacterial suspensions were aseptically transferred to the bottom of the tubes and then 2.0 ml volumes of sterile serum or banked blood were added. Transparent screw caps to which 1/4" disks of diazo black film had been attached to the inner surface were then tightly applied, leaving a head space between the liquid sample surface and the inner surface of the cap of approximately 3 cm. The sealed tubes were then incubated at 4^{o} C for varying lengths of time up to 12 days. The caps were then removed, the disks examined visually for a color change to black and then removed from the caps and placed in rows on clear polystyrene plates. Densities of the disks were then quantitated by Laser Scanning Densitometry at 200 nm resolution from 0 to 255 grey scale.

4. Results:

a. Screening Experiments in Microtiter Plates:

Bacterial suspensions in sterile saline, Tryptone broth, sterile fetal calf serum or 3 mM aqueous urea solutions were screened for their potential to evolve ammonia at levels sufficient for detection by a variety of commercially available ammonia-developing diazo papers or films. In these screens, uninoculated broth, sterile calf serum and sterile urea solutions served as negative controls. Urea solutions containing Jack Bean urease and calf serum containing urease served as positive controls.

Qualitatively, all of the bacterial species screened in this manner produced ammonia at levels sufficient to effect color changes of varying densities when mixed with calf serum. The color change densities observed from the bacterial-serum mixtures were generally significantly greater than those observed from the negative controls at both ambient room temperature and at 4^{o} C. While all of the ammonia sensitive papers and

films screened by this method were observed to detect ammonia to varying degrees of color density, Fast Speed Diazo Black film (FSDB) appeared to produce the most rapid and consistent response to the ammonia evolved from these bacterial-serum mixtures. For this reason FSDB was selected for use as a remote sensor in the cryovial test system for studies of Blood-bacterial interactions.

b. Cryovial Results:

1. Blood Spiked with Bacteria: A number of preliminary experiments utilizing 2.0 ml volumes of banked blood at bacterial contamination levels of approximately 10^3, 10^5 and 10^7 cfu/ml blood were completed to determine the validity of the test system. In general, the results observed during this preliminary work demonstrated that

1) the FSDB disks were sensitive to the levels of ammonia produced by the contaminated blood samples, both at ambient room temperate and at 4^{O} C and

2) the degree of color change exhibited by the FSDB disks could be quantitated by Laser Densitometry.

We also observed that uncontaminated blood (negative controls) produced levels of ammonia lower than those observed with the experimentally contaminated blood, but nonetheless, detectable by the FSDB sensor system. Greenwalt, *et al.* (7) have shown that low levels of ammonia are a normal product of red blood cell metabolism.

A major experiment was completed which involved deliberate contamination of banked blood with ten different species of bacteria at microbial levels of approximately 1 to 5 x 10^6cfu/ml of blood. These tubes, prepared in triplicate, were incubated in sets for 3, 6, 9 or 12 days at 4^{O} C and then examined visually and analyzed by Laser Densitometry. Because of the large number of tubes required, this study was carried out over two consecutive days and utilized blood from the same unit of blood on both days. Thus on Day 1 (series 1) blood was spiked with a set of 5 organisms and on Day 2 (series 2) blood from the same unit was spiked with a different set of 5 organisms. The results obtained from this experiment are as follows:

i. Eight of the ten selected bacterial species effected a color change in the diazo film which increased with time over 12 days and which in each case was statistically significantly greater than those observed for uncontaminated blood controls for all time periods (Table 2, Appendix pA2).

ii. Significant differences in red blood cell volumes were observed between the series 1 and series 2 sets of tubes. Where the number of samples totaled 54, the per cent settled red blood cells averaged 80% of total attributable to settled red blood cells. These observed differences in red during distribution of samples by gravity feed (Table 3, Appendix pA2).

iii. The two bacterial species which did not effect color changes in the sensor greater than those obtained with uncontaminated controls were *B. subtilis* and *P. vulgaris*. Both of these species were used to contaminate blood in the series 2 set of tubes which were subsequently found to contain a significantly lower red blood cell content than the series 1 set (Figures 1-3, Appendix, ppA4-A6).

2. Blood Spiked with Cell-Free Urease: Two different sources of urease, from a plant and a bacteria were tested for the ability to generate ammonia when added to whole blood. Increased amounts of both enzymes in blood produced resultant enhanced densities of sensor response as determined visually and instrumentally by laser densitometry. A double reciprocal plot of the quantitative data for each enzyme *vs.* response revealed an approximate linear relationship typical of enzyme saturation kinetics (Figure 4, Appendix pA7).

3. Blood Spiked with Ammonium Carbonate: Whole blood was spiked with commercially obtained and assayed ammonia solution (Sigma Chemical) starting at a concentration of 10 μM then two-fold serially diluted in blood to a 0.15 μM concentration in cryovials containing FSDB film disks. Quantitative data was obtained by Laser Densitometry. Statistical analysis of the triplicate samples indicated that the color obtained at every ammonia level was significantly different than blank controls. However, the zero control, with no added ammonia while significantly different than the blank was not different than those which changed in blood with added ammonia. There was, however a statistically significant linear trend for the mean density values *vs.* ammonia concentrations. It must be noted that the blood was drawn three weeks prior to use in the experiment. These results suggest that aging blood can change the color of FSDB sensor disks and the detection limit for this material may be greater than 10 μM.

The final Gantt chart showing all proposed work completed is found in the Appendix pA8.

(7) Conclusions:

A. SUMMARY:

1. Fast Speed Diazo Black Film, an ammonia sensor, is capable of detecting ammonia evolving from microbial metabolism in contaminated blood at levels greater than those evolved via red blood cell metabolism in uncontaminated controls at banked blood storage temperatures of 4^{O} C.

2. This sensor system does not require invasion of the blood bag unit for sample withdrawal. When exposed to ammonia, a common microbial metabolite, the indicator exhibits a readily apparent color change not requiring expensive equipment for interpretation and is easily assessed by non-technical personnel.

3. Quantitatively, the amount of ammonia produced in contaminated blood is the sum of that evolved by normal red blood cell metabolism plus that produced by the metabolic activity of the contaminating microorganism. Variability in hematocrit values from one unit of banked blood to another can be expected to lead to variations in the levels of ammonia detected in contaminated blood.

4. The results obtained from this Phase I effort provide scientific proof in support of the concept that an ammonia-sensitive sensor can be applied to the detection of microbial contaminants in banked blood.

5. Overall, work completed to date strongly suggests that this remote sensor system has a high potential for the eventual development of a viable product of continuing interest to the DoD and the private sector.

B. RECOMMENDED FUTURE WORK:

1. Limits of Detection-Microbiology. Additional work, employing lower levels of bacterial contamination at 10, 10^2, 10^3 and 10^4 cfu/ml blood is a critical extension of the work completed in Phase I.

Since *Y. enterocolitica* and *Pseudomonas* species account for 51% and 31%, respectively, of bacterial species associated with sepsis directly attributable to blood transfusions (1), it would be expedient to determine the lower limits of detection of contamination by the these organisms as an immediate first priority.

2. Hematocrit Effect. The effects of varying hematocrit levels on the selectivity of the sensor to distinguish between background levels of ammonia produced as a consequence of red blood cell metabolism and higher ammonia levels produced as a result of microbial metabolism in contaminated blood must be further characterized. Since it would be prohibitive to attempt to meet this objective on a statistically significant number of independent banked blood units, this objective might best be achieved by reconstituting red blood cells in plasma over a range of hematocrit values.

3. Limits of Detection-Sensor. The Diazo Black film, selected as the remote sensor during this Phase I effort, currently serves as the "Gold Standard" for initiation of future development. An additional effort is recommended to develop more sensitive diazo systems which might react more rapidly and or respond to extremely low levels of ammonia.

4. Qualitative Sensor Response. Explore sensor systems which may exhibit the capability to blank out or ignore low background ammonia levels formed as a result of red blood cell metabolism.

5. Blood Bag Engineering. Finally, efforts should be initiated towards engineering blood bags to which the sensor is affixed to the exterior of the bag, or is sensitive to gaseous ammonia that permeates the wall of the bag, and is protected against light destabilization and exhibits long shelf life stability.

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(9) Appendix:

TABLE 1

June 9, 1994

Selected Microbes and Ammonia-Producing Enzyme Activities.

ROW #	ORGANISM	ATCC STRAIN#	UREASE?	ARGDeHASE?	GRAM
1	PSEUDOMONAS AERUGINOSA	15442	-	+	-
2	STAPHYLOCOCCUS EPIDERMITIS	17917	+	+	+
3	KLEBSIELLIA PNEUMONIAE	12657	+	-	-
4	STREPTOCOCCUS FAECALIS	6057			+
5	ESCHERICHIA COLI	11229	-	-	-
6	STAPHYLOCOCCUS AUREUS	6538	+	+	+
7	BACILLUS SUBTILIS (spores)	9372	+	-	+
8	SERRATIA RUBIDEA	277593	-	-	-
9	PROTEUS VULGARIS	13315	+	-	-
10	YERSINIA ENTEROCOLITICA	9610	+	-	-

APPENDIX I

TABLE 2-STATISTICAL ANALYSIS

Bonferroni Multiple Comparison Test of Film Color Intensity Generated by Bacteria in Blood Compared to Control

	INCUBATION PERIOD			
ORGANISM	Day 3	Day 6	Day 9	Day 12
SERIES 1				
Pseudomonas aeruginosa	ns	ns	ns	***
Staphylococcus epiderm	ns	**	**	**
Klebsiella pneumoniae	ns	**	***	***
Streptococcus faecalis	ns	ns	ns	*
Escherichia coli	ns	ns	***	***
SERIES 2				
Staphylococcus aureus	ns	ns	ns	**
Bacillus subtilis	ns	ns	ns	ns
Serratia rubidea	ns	ns	***	ns
Proteus vulgaris	ns	ns	ns	ns
Yersinia enterocolitica	ns	ns	***	ns

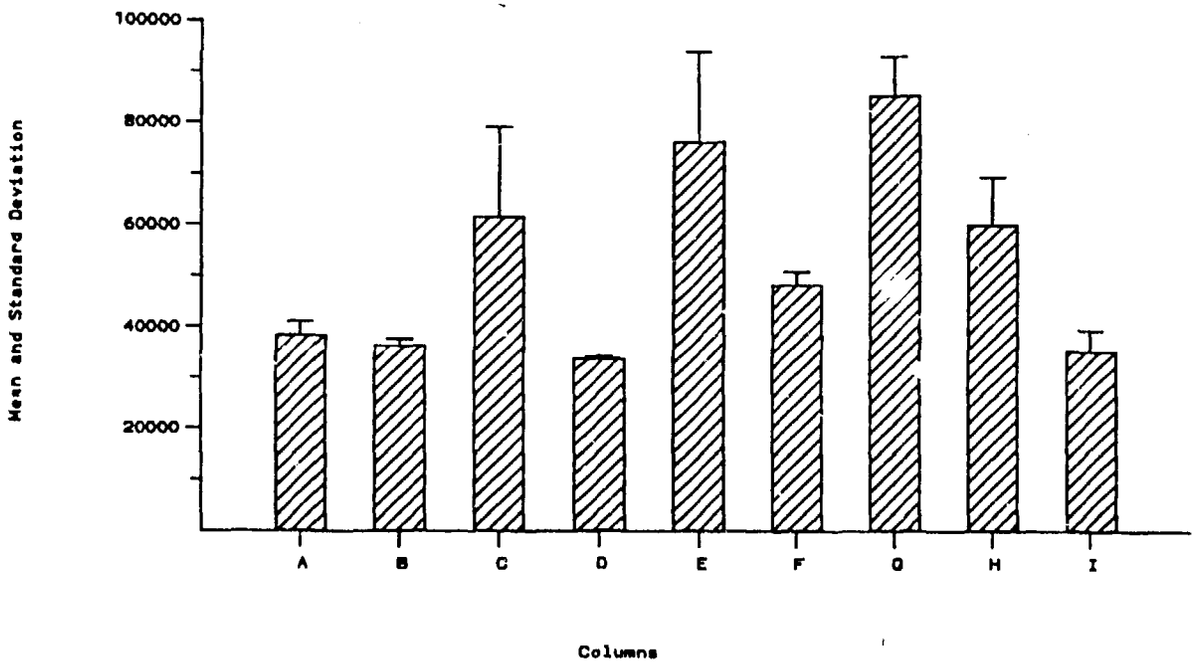
KEY: ns = not significant; * = p< 0.05; ** = p< 0.01; *** = p< 0.001

TABLE 3-RED BLOOD CELL VOLUMES

Percentage of Settled Red Blood Cells per Tube Volume for Two Series of Experiments and their Net Mean Color Intensities

	Percent of Volume, n=54	Pooled Net Change +SEM
Bacteria Series 1	80	22925 ± 3832
Bacteria Series 2	34	6346 ± 1617

FIGURE 1
SENSOR RESPONSE TO *S. epidermis* IN WHOLE BLOOD OVER 12 DAYS AT 4°C.



KEY:

A=DAY 3 EXPERIMENTAL
B=DAY 3 CONTROL

C=DAY 6 EXPERIMENTAL
D=DAY 6 CONTROL

E=DAY 9 EXPERIMENTAL
F=DAY 9 CONTROL

G=DAY 12 EXPERIMENTAL
H=DAY 12 CONTROL

I=BLANK VALUE

FIGURE 2

NET SENSOR DENSITY CHANGES-SERIES 1

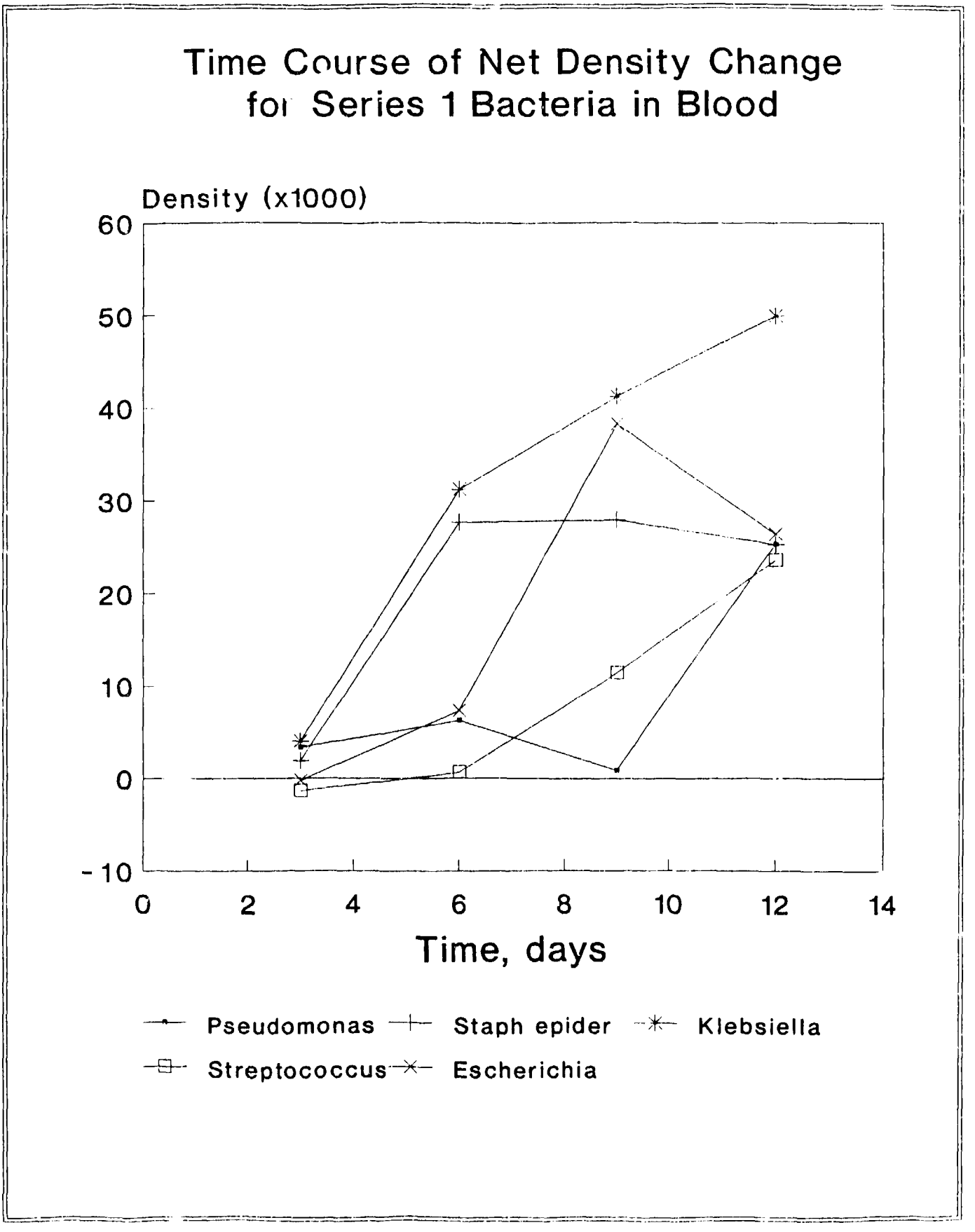


FIGURE 3

NET SENSOR DENSITY CHANGES-SERIES 2

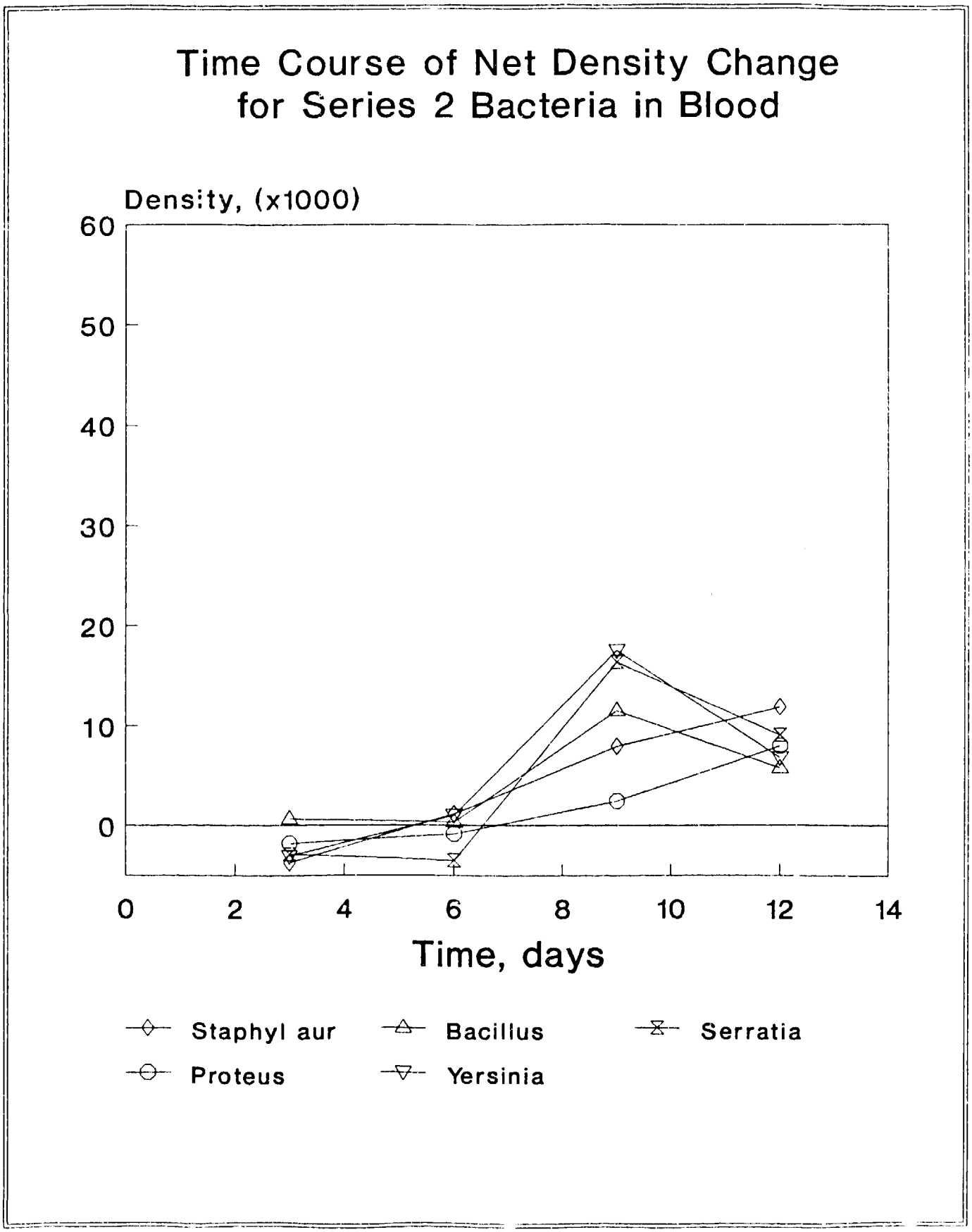
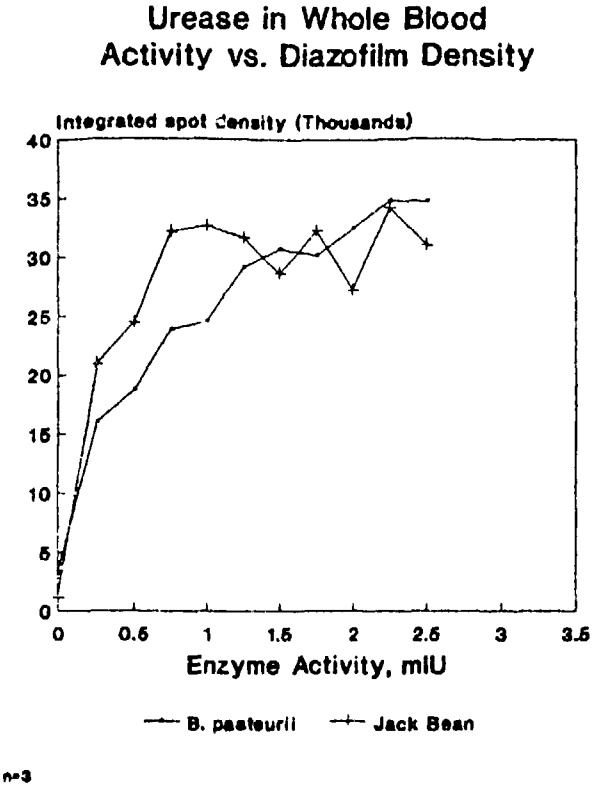
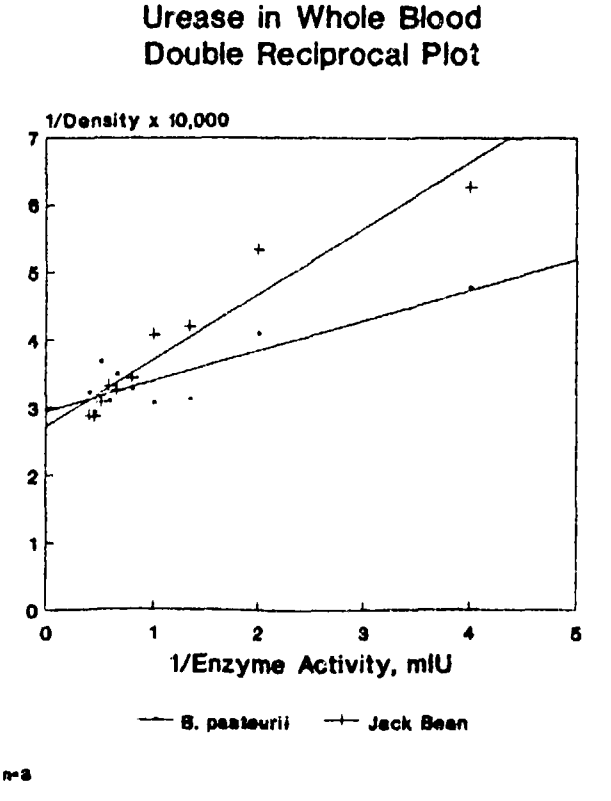


FIGURE 4

SENSOR DENSITY CHANGES *VS.* UREASE ACTIVITY IN WHOLE BLOOD





PHASE I GANTT CHART

Contract Number DAMD17-94-C-4038

"Systems to Detect Bacterial Contamination of Banked Blood in a Rapid, Non-Invasive Low Technology Manner"

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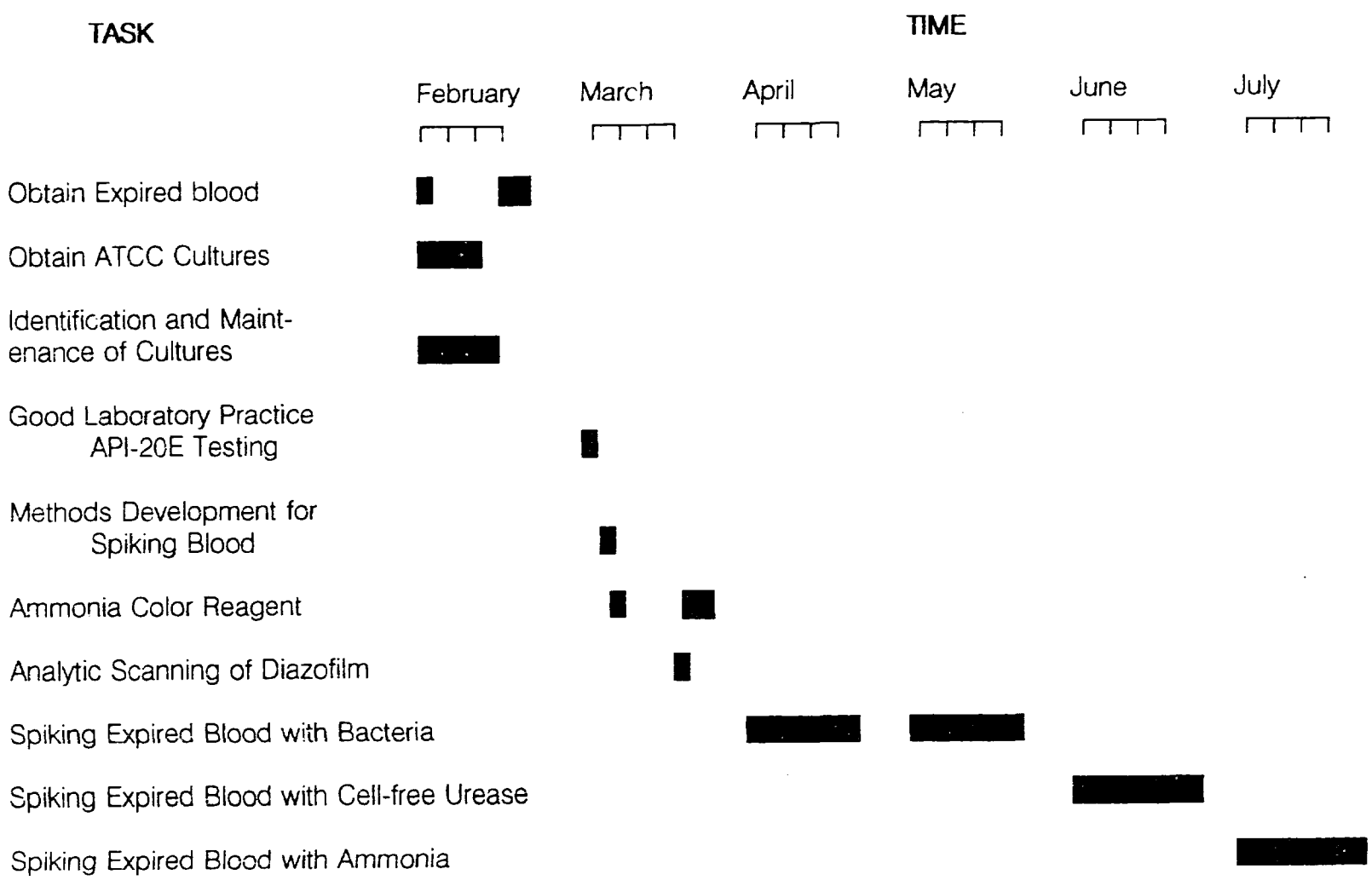

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